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Published in:
Physical Review Letters

DOI:
10.1103/PhysRevLett.74.2138

Publication date:
1995

Real Time Observation of Low-Temperature Protein Motions

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(Received 16 September 1994)

Optical methods were used to study the internal motions of myoglobin and cytochrome c. The experiments show that these proteins exhibit conformational fluctuations at temperatures as low as 2 K. The distribution of fluctuation rates can be measured in real time and turns out to be very sharp. The temperature dependence of the structural relaxation of myoglobin follows a simple Arrhenius law. The results are in agreement with existing models for protein dynamics.

PACS numbers: 87.15.–v

Proteins are a very interesting state of matter [1,2]. They exhibit properties that are reminiscent both of the crystalline state and of the glassy or liquid state. The crystalline properties of proteins are reflected in the fact that, in the native state, they have well-defined tertiary structures. However, proteins are not completely static systems. The tertiary structure should be considered as an average around which conformational fluctuations take place. Exploratory experiments by Frauenfelder, Petsko, and Tsernoglou showed that myoglobin can assume a large number of slightly different structures, conformational substates (CS's), separated by energy barriers [3]. Evidence for multiple potential energy minima also comes from molecular dynamics simulations [4]. The presence of multiple minima on the potential energy surface seems to be a general property of amorphous materials, which include proteins, glasses, and polymers. The anomalous properties of glasses have been successfully explained by the two-level-system (TLS) model [5,6]. A TLS comprises a group of atoms or molecules that can reside in either of two potential energy wells along a conformational coordinate. The TLS can flip between the two conformational states, by either quantum-mechanical tunneling or thermal activation. There is a strong similarity between the CS model for proteins and the TLS model for glasses. This is an interesting point, since glasslike behavior for proteins is suggested by both theory and experiment [4,7,8]. A characteristic property of proteins is that the CS's are organized in a hierarchy [9]. The idea is that within each substate of a particular tier in the hierarchy a number of sub-states exist, which comprise the next tier of CS's. The average height of the barriers between CS's decreases in descending tiers. At least three tiers of CS's exist for myoglobin [1]. In this Letter the focus is on a low tier of CS's with barriers between them that are still surpassable at temperatures nearing absolute zero.

Optical methods are very well suited to study the structural dynamics of amorphous materials [10–13]. The central idea behind the experiments presented in this Letter is that the optical resonance frequency of the porphyrin inside a heme protein depends on the conformation of the protein. Because each individual protein occupies a different CS, the optical spectrum of an ensemble of chromoproteins is inhomogeneously broadened. However, the absorption frequencies of individual chromophores are not necessarily constant. If a protein flips from one CS to the other, the porphyrin will respond by a shift of its resonance frequency. This effect is commonly referred to as spectral diffusion. The internal motions of heme proteins can be studied by monitoring the spectral diffusion of the porphyrin.

Several different techniques are available to study spectral diffusion. The experiments presented here are three-pulse stimulated photon echoes (3PSE's). Although the 3PSE is a time domain experiment, its essential features can be clarified within the frequency domain [14]. In the 3PSE experiment the sample is excited by three laser pulses that overlap in space but do not overlap in time. The pulses have a typical duration of 5 ps and a typical bandwidth of 8 cm^{-1}. Their frequency is resonant with an optical transition of the chromophore. The first two pulses, which are separated by a time interval τ, generate a frequency grating within the spectrum of the chromophore. A frequency grating is a modulation of the population of chromophores in the ground state and in the excited state as a function of their resonance frequency. The fringe spacing of the frequency grating is equal to $1/\tau$. The essential point is that by creating a frequency grating we label chromophores with respect to their resonance frequency and therefore with respect to the conformational state of the protein. Just as an optical pulse can be scattered from a spatial grating, the third pulse, which is delayed with respect to the second pulse by a waiting time t_W is scattered from the frequency grating as the photon echo. What is important is that the intensity of the echo signal is proportional to the modulation depth of the frequency grating. Figure 1 demonstrates the effect of spectral diffusion on two different frequency gratings: a coarse grating, generated by two pulses separated by a short time τ, and a fine grating, generated by two pulses separated by a long time τ. Spectral diffusion reduces the modulation depth

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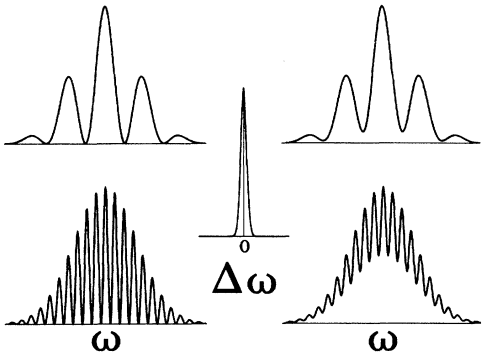

FIG. 1. Schematic representation of the effect of spectral diffusion on two frequency gratings. Shown in the center is the distribution of frequency shifts experienced by individual chromophores during a certain waiting time. The two gratings on the right-hand side result from the effect of these frequency shifts on the two gratings on the left-hand side. The modulation depth of the fine grating is reduced much more strongly than that of the coarse grating, resulting in a weaker photon echo signal.

of both gratings. However, the net effect strongly depends on the fringe spacing and therefore on τ. As a consequence the decay rate of the photon echo intensity as a function of τ, k_{echo}, will increase when spectral diffusion occurs during t_W.

Structural dynamics in amorphous systems can cover a broad range of time scales. The dependence of k_{echo} on t_W can reveal the particular time scales that are important. At this point it is convenient to introduce a distribution function $P(R)$ of fluctuation rates R. It is assumed that transitions between conformational states occur instantly and the fluctuation rate is defined as the inverse of the average time between them. The main merit of the experimental setup is that t_W can be continuously varied between 0 and 100 ms, allowing the study of spectral diffusion in real time [15]. In this way we obtain information about $P(R)$, which can be related to the structure of the potential energy surface of the system under investigation. A theoretical description of spectral diffusion in magnetic resonance experiments was provided by Hu and Walker [16]. Bai and Fayer extended their results to optical experiments [17]. Based on this work we can relate the dependence of k_{echo} on t_W to $P(R)$ in the following way:

$$k_{\text{echo}}(t_W) = k_{\text{echo}}(t_W^0) + \int_0^\infty dR\, P(R)[\exp(-Rt_W^0) - \exp(-Rt_W)], \tag{1}$$

with $t_W > t_W^0$. The integral in Eq. (1) should be considered as the effect of spectral diffusion occurring between t_W^0 and t_W. By fitting the experimental data to Eq. (1), $P(R)$ can be determined. Previous experiments have shown $P(R)$ to be broad and featureless for organic glasses and polymers [18,19].

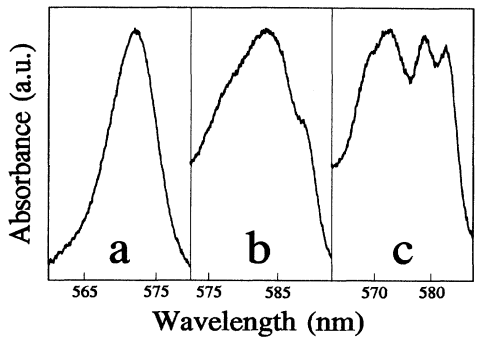

FIG. 2. Optical absorption spectra of (a) Zn-MP in 3/1 volume glycerol/dimethylformamide at 77 K, (b) Zn–cyt c in 3/1 volume glycerol/10 mM phosphate buffer pH 7.0 at 95 K, and (c) Zn-mb in glycerol/buffer at 77 K,

The systems studied were Zn-substituted cytochrome c (Zn–cyt c) and the 1:1 Zn–mesoporphyrin IX:apomyoglobin complex (Zn-mb). The chromophore in both systems is Zn–mesoporphyrin IX (Zn-MP). The proteins were embedded in a host glass of glycerol and phosphate buffer (pH 7). Figure 2 shows optical absorption spectra in the Q (0-0) region for both systems along with a spectrum of Zn-MP directly dissolved in a glassy matrix of glycerol and dimethylformamide. These spectra show that the optical properties of the chromophore are strongly influenced by its local environment. All spectra are broadened due to inhomogeneities in the local sites experienced by individual chromophores, which is typical of spectra of chromophores embedded in amorphous materials. However, we observe remarkable differences between the glass and the protein. The spectrum of Zn-MP is smooth and featureless when it is directly dissolved in the glass, whereas bound to a protein it shows substantial substructure. This is due to the taxonomic nature of the highest tier of CS's. For myoglobin it is known that there are three CS's on top of the hierarchy [1], which accounts for the three distinct bands in the absorption spectrum.

Figure 3 shows k_{echo} vs t_W for Zn–cyt c at 1.8 K and for Zn-mb at 1.9 K. Also shown in Fig. 3 are the distribution functions used to fit the data to Eq. (1). It appears that spectral diffusion occurs in two distinct regions of t_W, implying the existence of at least two distinct distributions of fluctuation rates. We tentatively assign the increase of k_{echo} in the submicrosecond region to coupling of the porphyrin to the TLS's of the glycerol/water glass. Because the protein shields the chromophore from direct contact with the host glass, these TLS's must be intrinsic to the glass. The distinction between intrinsic and extrinsic TLS's of glasses was emphasized by Jankowiak and Small [20]. The coupling between the chromophore and the host glass is accounted for by a hyperbolic distribution function that cuts off at $\log_{10}R \approx 6.3$. Coupling between the porphyrin and the host glass has been observed before [21] and we can

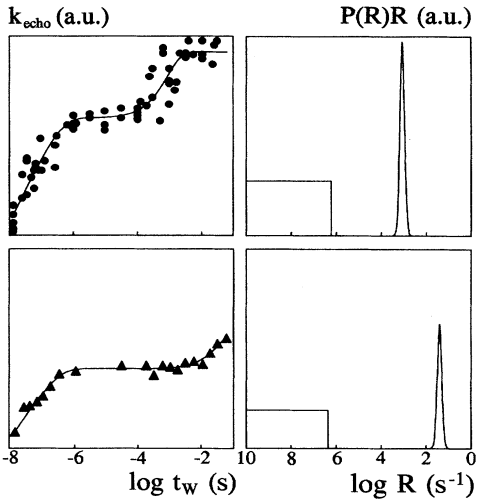

FIG. 3. k_{echo} vs t_W for Zn–cyt c at 1.8 K (●) and Zn-mb at 1.9 K (▲). The solid lines through the data are fits to Eq. (1) using the distributions displayed on the right-hand side.

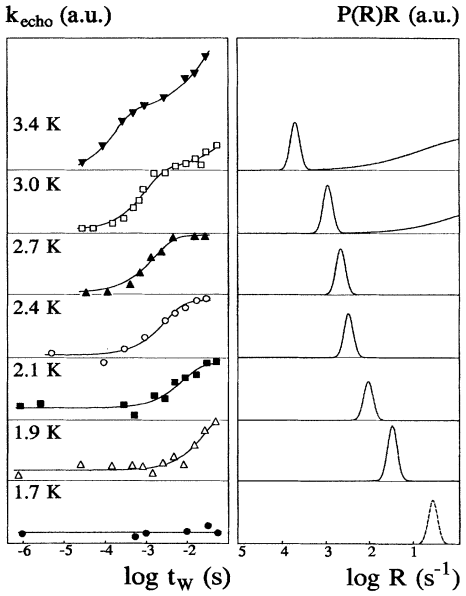

FIG. 4. k_{echo} vs t_W for Zn-mb at 1.7 K (●), 1.9 K (△), 2.1 K (■), 2.4 K (○), 2.7 K (▲), 3.0 K (□), and 3.4 K (▼). The solid lines through the data are fits to Eq. (1) using the distributions displayed on the right-hand side.

rationalize the smaller slope for Zn-mb in terms of a more effective shielding of the porphyrin. The special merits of hyperbolic distribution functions to describe spectral diffusion induced by flipping of TLS's in glasses have been discussed elsewhere [12,18,19].

The second increase of k_{echo} occurring in the ms region starts at much longer t_W for Zn-mb than for Zn–cyt c. The chromophore, the host glass, and the temperature are comparable for both sets of data. Hence the observed difference can only be explained by the fact that the bulk protein is different. Therefore we assign the second increase of k_{echo} to conformational fluctuations of the protein. The fact that k_{echo} levels off at $t_W \approx 3$ ms for Zn–cyt c demands a narrow range of fluctuation rates. The necessity of a sharp distribution becomes even more apparent when we study the temperature dependence for Zn-mb. Figure 4 displays k_{echo} vs t_W for Zn-mb at seven different temperatures between 1.7 and 3.4 K together with the distributions used to fit the data. We can clearly see that the sharp distribution shifts towards faster fluctuation rates.

How do we relate these observations to the internal motions of the protein and the structure of its potential energy surface? First of all a sharp distribution of fluctuation rates and therefore of barrier heights favors a hierarchial arrangement of CS's. A different argument considers the magnitude of the frequency shifts induced by flipping between CS's. The spectral shifts that cause the increase of k_{echo} are smaller than one-tenth of a wave number. From the absorption spectrum of Zn-mb we can see that the central resonance frequencies of porphyrins in each of the three CS's of highest tier are at least several tens of wave numbers apart. Since the spectrum appears as a broad and relatively smooth band, it is obvious that there must be many tiers of CS's between the highest tier and the tier that is probed by the 3PSE experiment. Within this context it is very interesting that a second distribution is activated above 3 K. It is tempting to assign this distribution to the next tier of CS's.

A second important point concerns the width of the distribution. Because of scatter in the data the actual width of the distribution is uncertain. An indication that the actual distribution is sharper than those displayed in Fig. 4 is that no observable change of the width occurs with temperature. This would be expected since the position of the distribution depends strongly on temperature. A possibility that should be considered is that the distribution is infinitely sharp. This would imply that each protein comprises a single TLS. Measurements of the specific heat of metmyoglobin crystals also indicated that only a limited number of TLS's are active at low temperatures [7]. Such a view is in contrast with previous experiments on the higher tiers of CS's of myoglobin. These experiments showed that a taxonomic approach is only appropriate for the first tier. The lower tiers are believed to consist of many states and a statistic approach seems more appropriate [1].

The limited width of the distribution provides a unique opportunity to determine the thermodynamic parameters for conformational barrier crossing of a protein at rest. It is believed that the flipping of TLS's in glasses at low temperatures occurs through tunneling mechanisms. Barrier tunneling was also proposed to occur in proteins at temperatures as high as 40 K [22]. Figure 5 shows the natural logarithm of the fluctuation rate obtained from Fig. 4 vs the inverse temperature. The temperature

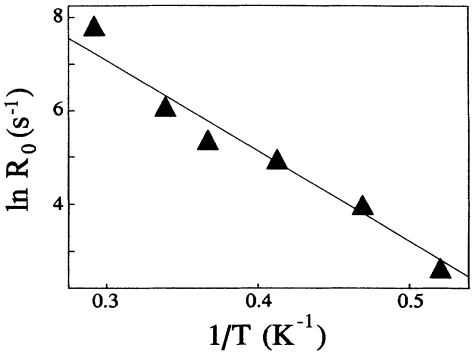

FIG. 5. $\ln R_0$ vs $1/T$ for Zn-mb. R_0 is half the fluctuation rate corresponding to the center of the distributions of Fig. 4. The factor $\frac{1}{2}$ reflects the fact that the fluctuation rate is defined as the sum of the probabilities of both "forwards" and "backwards" transitions between two states. The solid line is a linear fit to the data.

dependence is by no means consistent with a tunneling model but fits very well to a simple Arrhenius law, yielding an activation energy of 0.16 kJ mol^{-1} and a preexponential factor of 4.0×10^5 s^{-1}.

Although the experiment clearly demonstrates the presence of conformational fluctuations of the protein, it does not explicitly reveal the nature of these fluctuations. However, on the basis of the thermodynamic parameters some tentative conclusions may be drawn. The very small activation energy suggests that we deal with motions involving only very small mean displacements of atoms. The small preexponential factor makes us believe that the conformational fluctuation involves the motion of many atoms, possibly the whole protein. This is also supported by the fact that we observe a different fluctuation rate for Zn-mb and Zn–cyt *c* under similar conditions. The latter conclusion seems to be of importance in understanding the origin of a hierarchial arrangement of CS's. It suggests that each tier of substates involves motions of the whole protein, the difference being the mean displacements of atoms, as opposed to assigning different tiers to motions of different parts of the protein. Another important question is whether the behavior observed here is general for proteins. Hole-burning experiments by Friedrich *et al.* showed that distributions of limited width also apply to horseradish peroxidase, although at much higher temperature (>10 K) [23]. Obviously, a proper answer to this question can only be obtained from an investigation of a larger number of proteins.

Finally, we would like to make some concluding remarks concerning the relationship between proteins and glasses. It is now clear that proteins exhibit conformational fluctuations at temperatures as low as 2 K and in that respect they are similar to glasses. The similarity comes from the fact that the potential energy surface of both proteins and glasses is multidimensional and exhibits multiple minima. The main difference between proteins and glasses is reflected in the distribution function of fluctuation rates. The distributions for myoglobin and cytochrome *c* are much sharper than those observed for glasses and polymers. The reason for this difference lies in the detailed structure of the potential energy surface. The hierarchial arrangement of the CS's of proteins is consistent with an ultrametric structure of the conformational phase space. This ultrametricity does not seem to apply to glasses.

We gratefully acknowledge Professor J. M. Vanderkooi, Dr. A. A. van Dijk, and Professor G. T. Robillard for their assistance with the sample preparation. We are indebted to F. de Haan for providing us with software for data acquisition and handling. This work was supported by the Netherlands Foundation for Physical Research (FOM) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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